Germination-Lysis for Wide-Area Decontamination of *Bacillus anthracis* spores

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14. ABSTRACT
This presentation covered the use of a Germination-Lysis approach to decontamination of Bacillus anthracis spores, based on the fact that germinated spores are more susceptible to simple disinfection methods such as UV and desiccation. The presentation covered challenges to wide-area spore decontamination, challenges to germination-lysis decontamination, proof of concept, project focus, experimental approach, and the relative cost.

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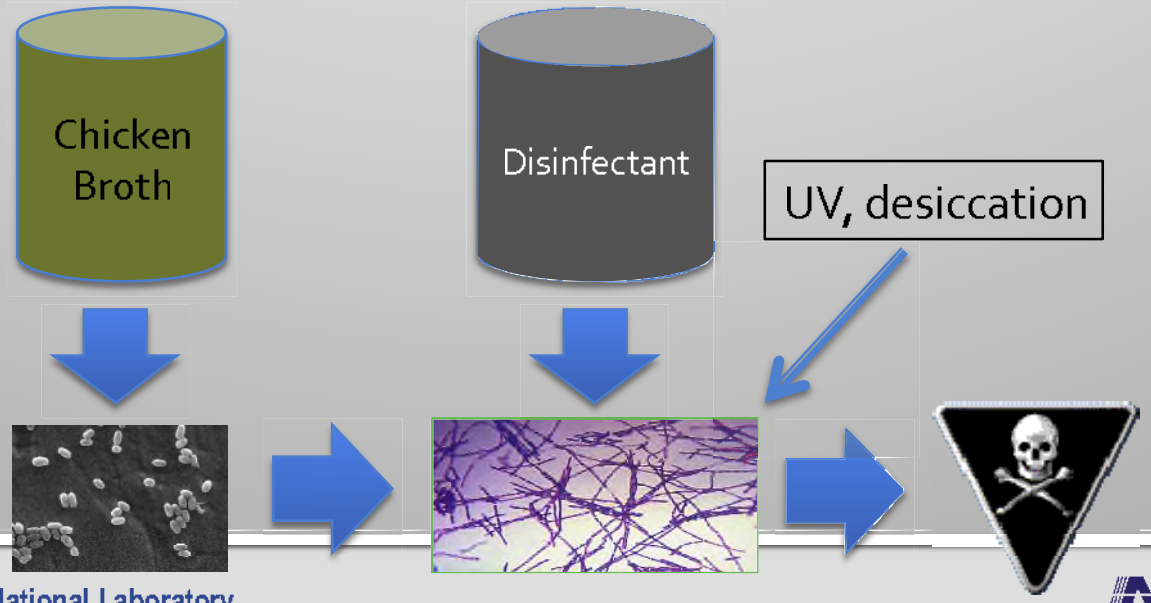
Challenges to Wide-Area Spore Decon

- Levels of disinfectant needed for spore lysis
- Disinfectants are corrosive, damaging to surfaces/ materials
- Disinfectants may be consumed in organic/chemical backgrounds in the environment
- Spore transport in liquid or air phase (reaerosolization), or fomites
- Incomplete decontamination
- Long-term human health and environmental impacts

Germinated spores and vegetative cells are more easily disinfected than bacterial spores

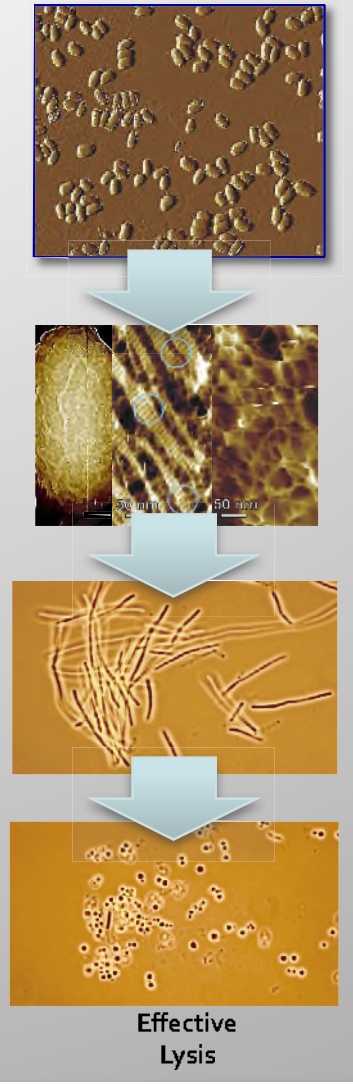
At acceptable environmental conditions and on appropriate surfaces, spores are germinated and then disinfected. Germinated spores and vegetative cells are more susceptible to simple disinfection methods, including UV exposure and desiccation.

- **Option 1**: Apply germinant and wait 15 to 30 min and then apply disinfectant solution (or demonstrate natural attenuation)
- **Option 2**: Apply germinant with disinfectant solution (*if feasibility is demonstrated*)

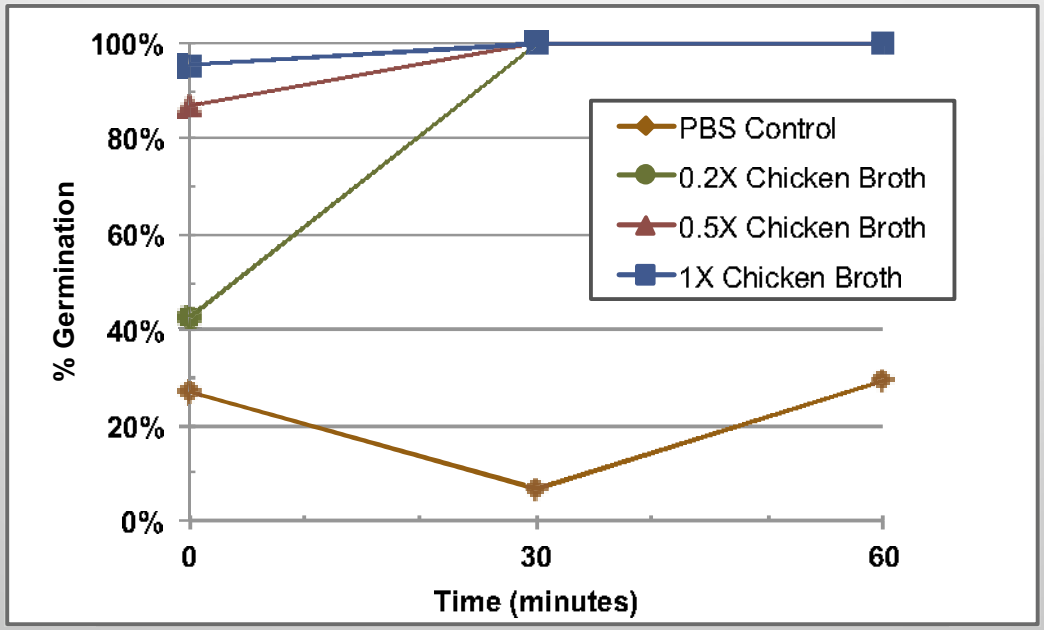


Challenges to Germination-Lysis Decon

- Levels of germinants and disinfectants needed
  - Materials may be consumed in organic/chemical backgrounds in the environment
- Maintenance of optimal environmental conditions for activity
- Kinetics of processes relative to ability to maintain optimal conditions
- Impact of nutrient source on indigenous populations (biofouling)
- Spore transport (liquid or air phase)
- Incomplete germination (dormant spores)
- Incomplete lysis—propagation of pathogen cells/ spores

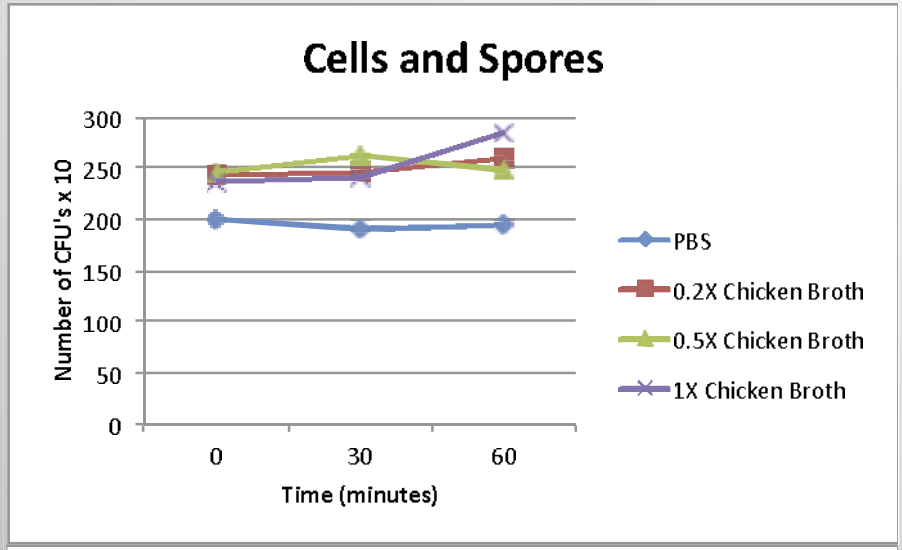


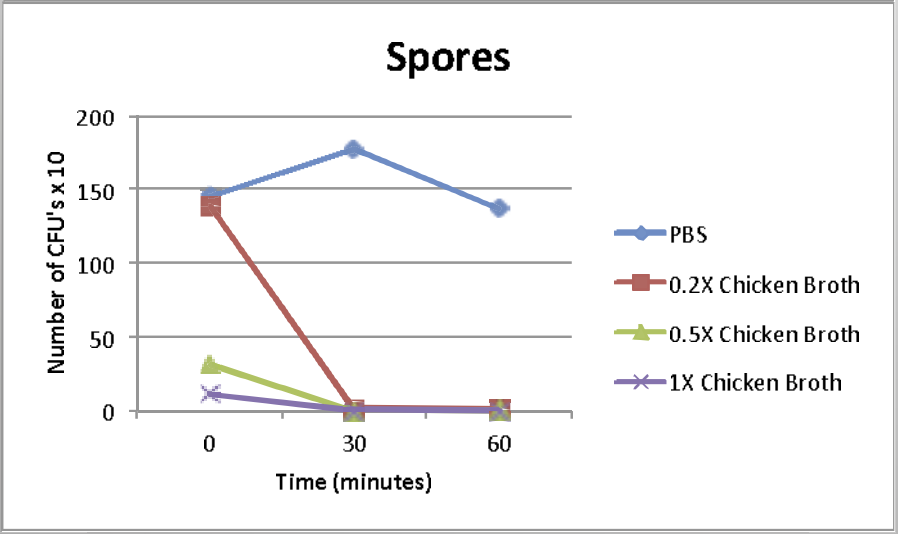
Proof of Concept: Low cost, readily available materials may be effective for spore germination



- Percent spore germination determined by heat treatment relative to the total population (cells and spores) from plate culture analysis (starting with 10^3-10^4 spores/mL).
- Filtered, fat-free chicken broth was diluted in phosphate-buffered saline.
- Time 0 represents 10-15 min exposure before analysis could be conducted.

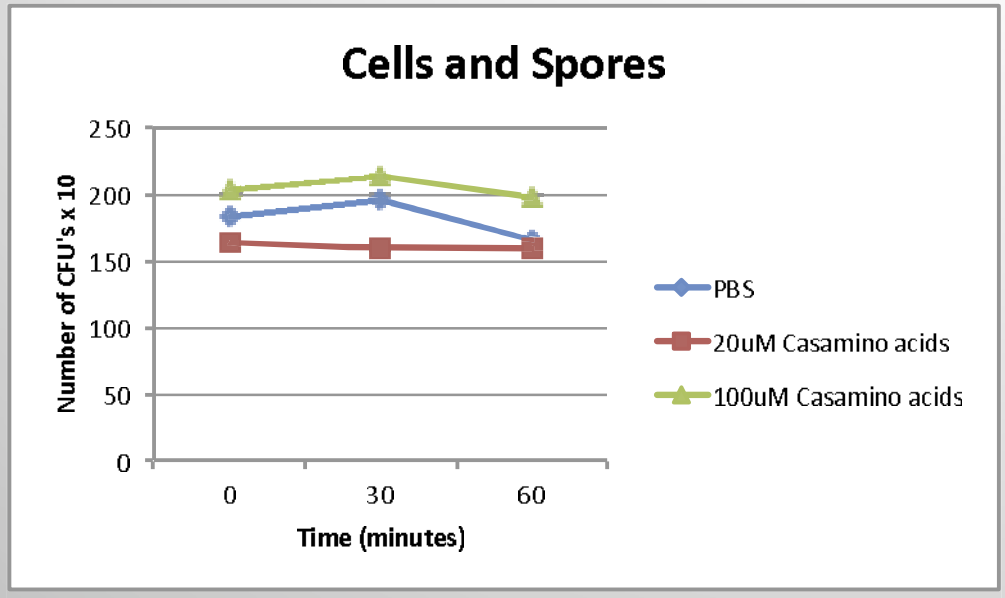
B. anthracis Sterne spores showed rapid germination with dilute chicken broth

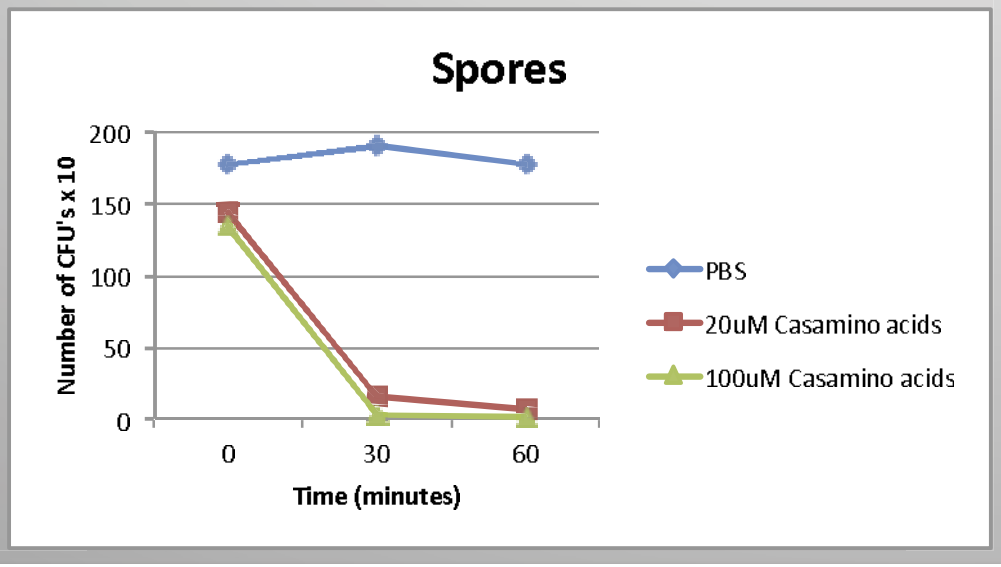




- Rapid germination immediately after broth addition (relative to control, phosphate-buffered saline, PBS)
- Nearly 100% germination at 30 min with 0.2X, 0.5X and 1X broth
- Average of 3 replicates shown
- Determined by direct plating for cells and spores
- Determined by heat treatment (65°C for 20 min) for spores
- Estimated material cost = $1.49 per L (1X) to $0.30 per L (0.2X) $5.60/gallon (1X) to $1.13/gallon (0.2X)

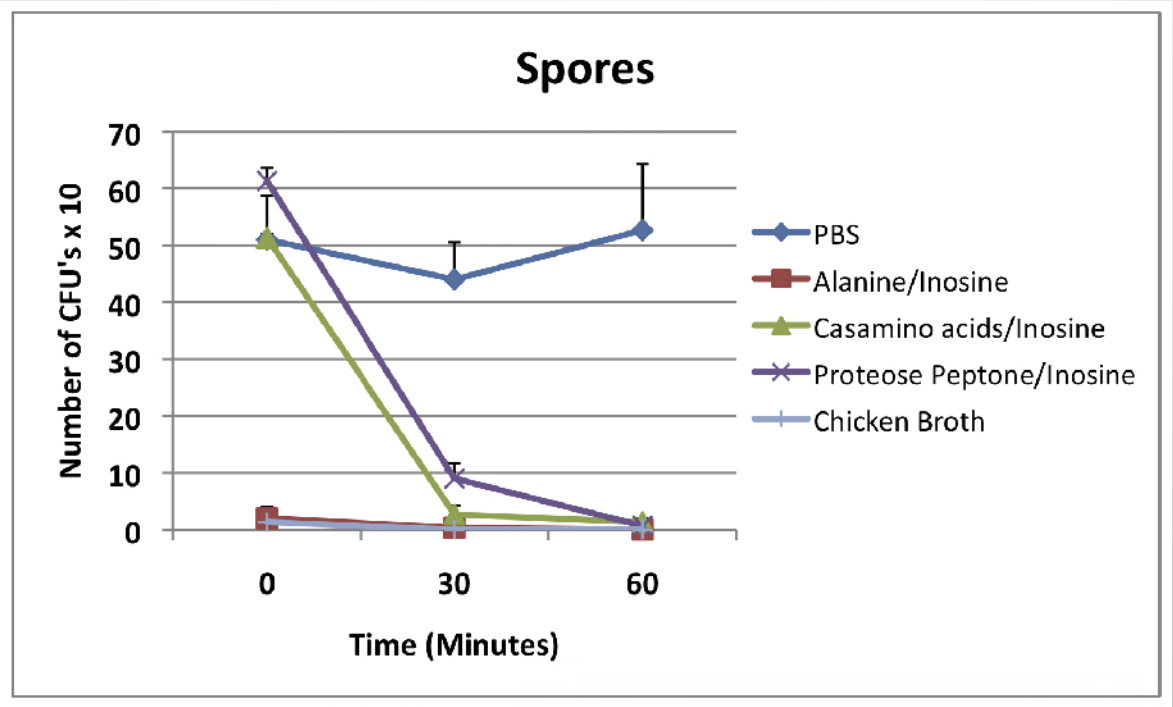
Dilute media components showed rapid spore germination





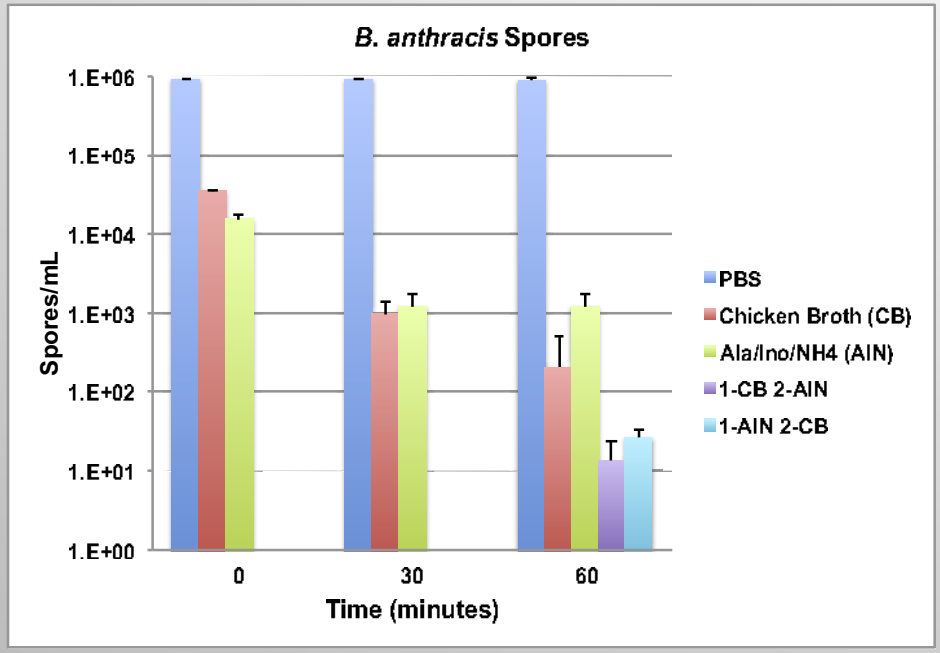
- Nearly 100% germination at 30 min with 20 µM casamino acids/100 µM inosine
- Average of 3 replicates shown
- Determined by direct plating for cells and spores
- Determined by heat treatment (65°C for 20 min) for spores
- Estimated material cost = $0.016 per L ($0.06/gallon)
- Lower cost for L-alanine, inosine – supplements

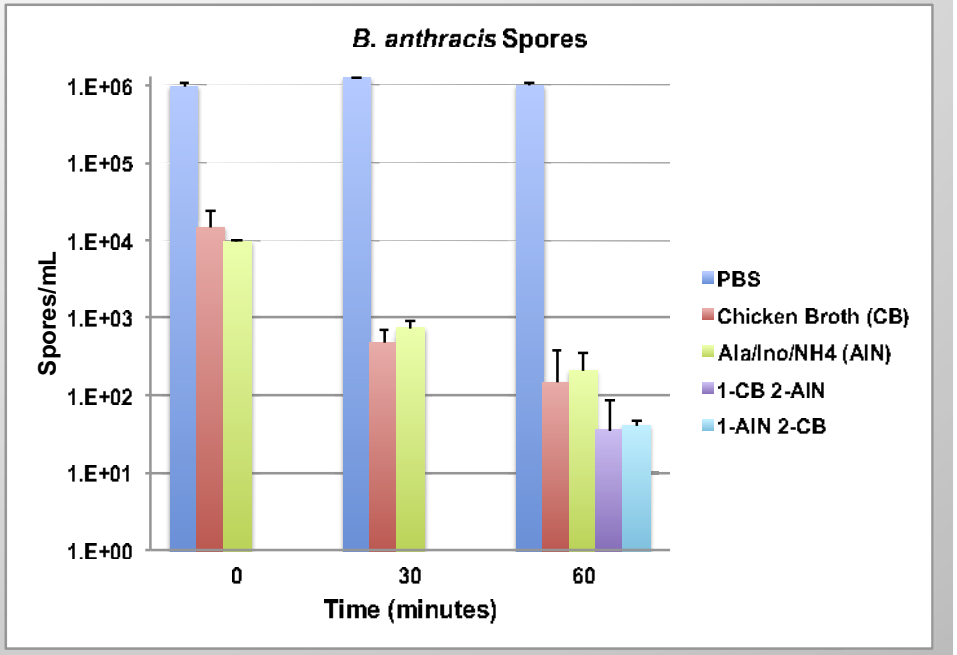
Summary: *B. anthracis* Sterne spores treated with simple germinant solutions



- Chicken Broth and L-alanine/inosine effective in first 15 min (time to process samples) for low spore levels (10^2 to 10^3)
- Casamino acids/inosine and Proteose Peptone/inosine effective in ~30 min
  - Casamino acids: 2% free Alanine; Proteose Peptone: 0.5% free Alanine; Inosine concentration not known

Subsequent addition of another germinant may enhance germination



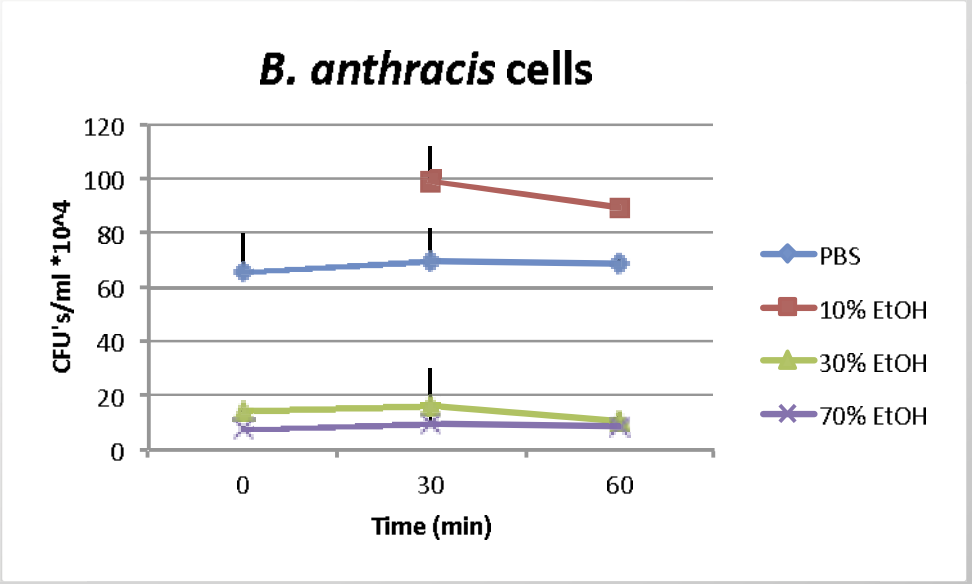


- 10^6 *B. anthracis* Sterne spores; 1X filtered, fat-free chicken broth (CB)
- Addition of alanine/inosine/NH_4Cl (AIN) to spores in CB or addition of CB to spores in AIN showed higher percent germination
- Need to compare with addition of same germinant
- Results for 30°C and 25°C were consistent; > 4 log germination

B. anthracis cells treated with lysis materials

Preliminary results showed:

- 30% and 70% ethanol were effective within 15 min
  - 10% was less effective
- 5% pH-adjusted bleach was effective within 15 min (100% lysis)

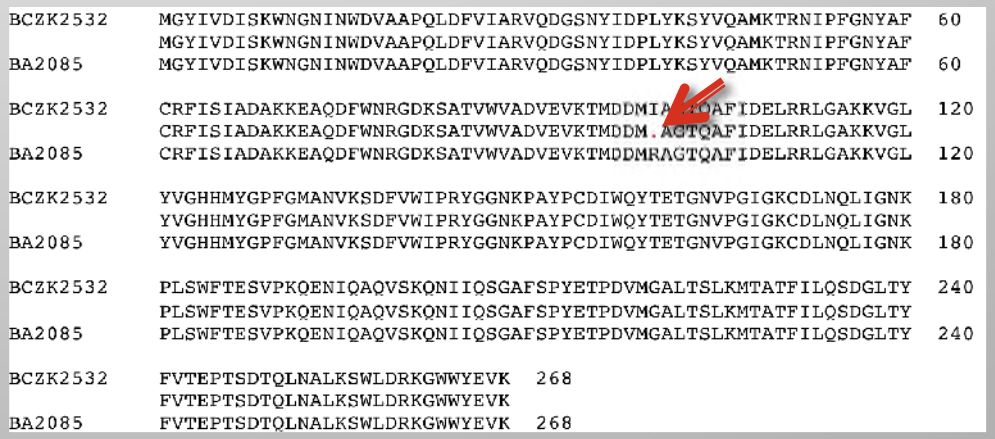


- 3% H_2O_2 was effective after 30 min (100% lysis)
- Salt up to 30% was only partially effective at cell lysis
- 5% acetic acid (vinegar) was ineffective

We are also investigating killing of vegetative cells due to natural degradation processes – desiccation and UV exposure

Bacterial lytic proteins may have utility for rapid cell lysis following forced germination

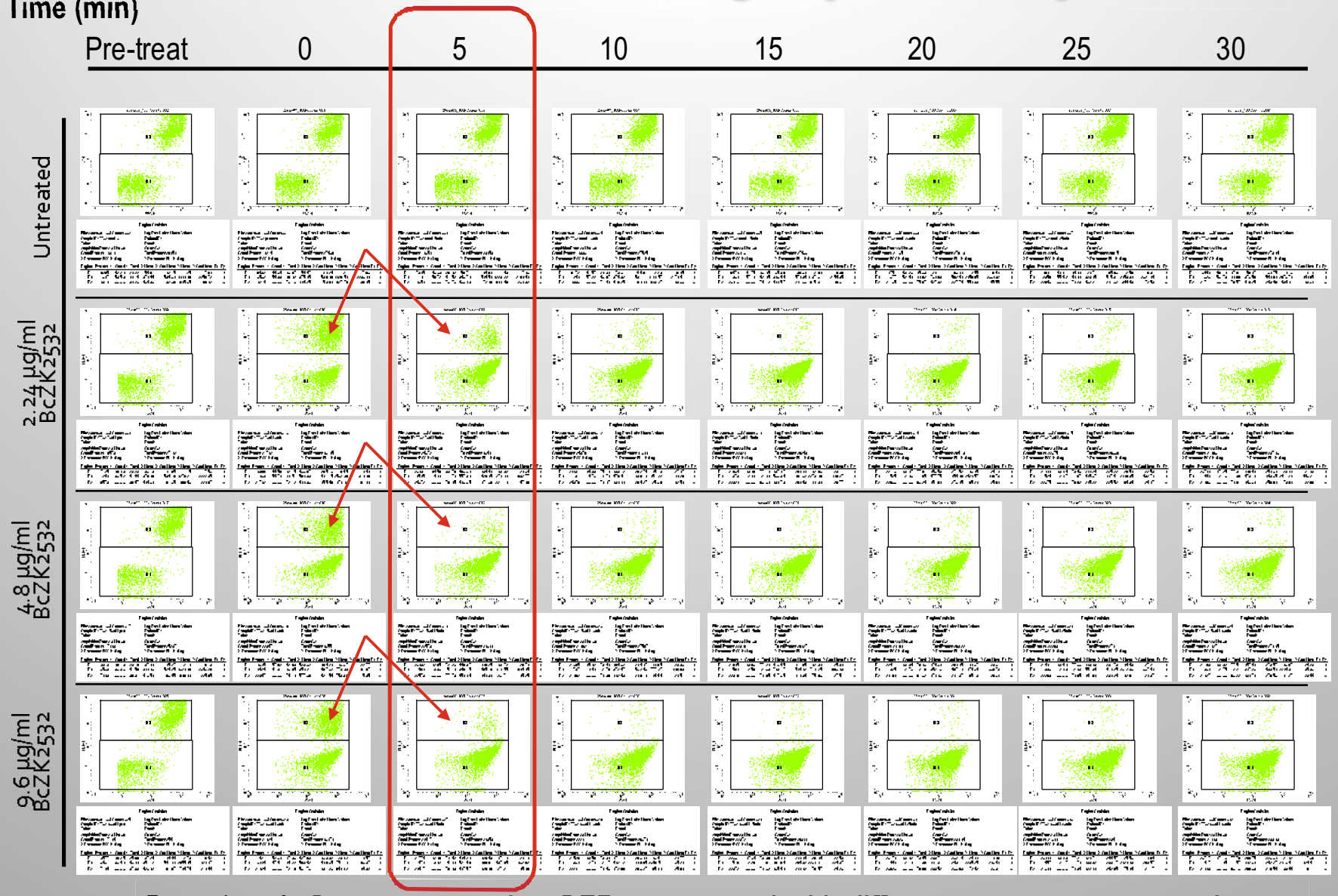
- Bacteriophage endolysins have been extensively studied as anti-microbial agents
- A less studied group of lytic proteins implicated in bacterial cell wall biosynthesis and recycling is encoded by all bacterial genomes
- This group consists of different protein classes differentiated by the specific cell wall component that they attack
- Bacterial lytic proteins show specificity for a relatively small group closely related microbes
- One promising candidate, AmpD (N-acetylmuramoyl-L-alanine amidase) from *Bacillus cereus* E33L (BcZK2532), a close relative of *B. anthracis*
  - ~100% identical to putative protein in *B. anthracis*



B. anthracis Sterne cultures begin to clear after amidase treatment

- Cells were treated with 300 nM (~9 µg/mL) amidase, incubated 35ºC for 15 min
- Amidase stable over 8 months at 4°C, with no special protection
- Large preparations of lyophilized BcZK2532 amidase showed no detectable loss of activity over 18 months

Recombinant Amidase Shows High Lytic Activity in 5 Minutes



B. anthracis Sterne expressing GFP was treated with different enzyme concentrations—low amidase (100 nM) concentrations were effective

Project Focus

- Investigation of low-cost germination-lysis approaches
  - Determine kinetics and extent of germination and lysis
- Evaluation of down-selected germination-lysis approach under relevant environmental conditions including temperature, relative humidity, and matrix interferences
  - Determine kinetics and extent of germination and lysis
- Investigation of application methods for optimal activity
  - Use of emulsions to maintain sufficient moisture content for germinant and disinfectant activities
  - Combine germination and lysis experiments after separate process optimization
- The influence of matrix interferences such as surface debris and indigenous microbial populations on both germination and disinfection
- Germination and lysis rates for surrogates evaluated in parallel with a virulent *B. anthracis* strain (Ames)

Collaboration

- **LLNL**
  - Dr. Joe Tringe: Engineering and spray technology for related applications
  - Dr. Alex Malkin: Spore ultra-structure analysis (AFM)
  - Dr. Sonia Létant, Select Agent Facility: Testing with virulent *B. anthracis*
- **University of California-Davis**
  - Dr. Ken Giles
  - Optimization of germinant and disinfectant application (formulation and delivery parameters) (follow-on effort)
- **US EPA National Homeland Security Research Center (NHSRC):**
  - Dr. Shawn Ryan, Dr. Worth Calfee
  - Experimental design and data analysis of microcosm studies and EPA chamber studies

Agricultural spraying can provide scale-up

- Liquid application rates: 10^{-3} to 1 L/m^2
- Deposition layers: 1 to 10^3 µm
- Work rates: 3 to 600 ha/hr
- Droplet size: 10 to 10^3 µm diam.
- Droplet velocities: 10^{-1} to 10^1 m/s

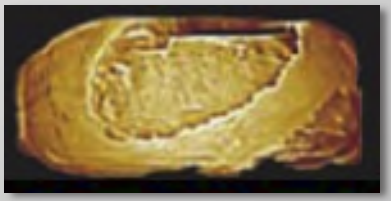

http://www.kirtland.af.mil/photos/mediagallery.asp?galleryID=2467

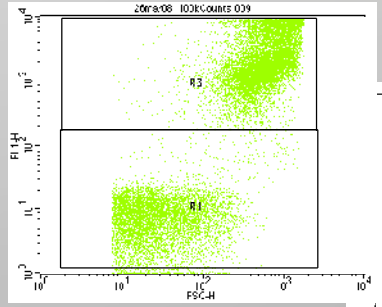

http://www.aero-news.net/index.cfm?do=main.textpost&id=07a7bbca-6124-4eae-ab7f-94fg806c9cco

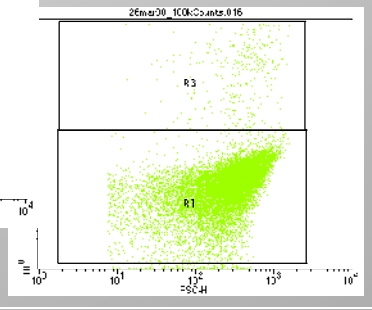

http://www.spraycontractor.com/

Summary: Experimental Approach

- Evaluation of surrogates relative to virulent strain for microcosm-level and scale-up studies (LLNL Select Agent Facility)
- Mass-balance for microcosm and chamber studies
- Evaluate 6-log level for germination and lysis (decon)
- Capability to include enzymes for forced germination and lysis
- Capabilities for structural analysis (AFM) for mechanistic information
- Flow cytometry for detailed kinetics studies (temperature, germinant conc., challenges)
- Collaboration for chamber and field scale-up of technology (UC-Davis, US EPA)
- High throughput viability testing capability
- Technology/Data Transition Agreement with US EPA







Preliminary results show promise for germination-lysis approach

Relative cost comparison

- Chicken broth estimated material cost = $5 to $11 per gallon germination solution (0.5x), ($2 to $5 at 0.2x solution)
- Casamino acids estimated material cost = $0.06 per gallon germination solution
- Alanine/Inosine estimated material cost = $0.05 per gallon germination solution
- Clorox bleach estimated material cost = $4 to $9 per gallon decontaminant solution

Acknowledgments

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- Sonia Létant, Gloria Murphy, Teneile Alfaro (Select Agent Facility LLNL) – confirmation with virulent *B. anthracis* strain
- Jessica Wollard (LLNL) – Technical support
- Joe Dalmasso – Apex Laboratories (Yakibou, Inc.), spore preparations